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Antimicrobial Activity of Electrochemical Silver Ions in Nonionic Surfactant Solutions and in Model Dispersions

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Abstract

The microbicidal effectiveness against Gram-positive and Gram-negative bacteria and *Candida albicans* of electrochemical silver ions in aqueous solutions containing nonionic surfactants was investigated. From the perspective of the possible use of anodic silver as a preservative in cosmetic or pharmaceutical preparations, microbicidal efficacy was also studied in oil/water model dispersions.

Surfactants and botanical extracts partially inhibited the microbicidal effectiveness of anodic silver. Nevertheless in all the experimental conditions, silver ions reduced the microbial concentration up to 4 log units of the starting inoculum in less than 6 h.

The wide microbicidal spectrum and the high rate of kill of silver ions appear, therefore, attractive enough to suggest a possible utilization of anodic silver as a preserving agent.

The preservation against microbial contamination of cosmetic and pharmaceutical water-containing preparations is an unresolved problem (Tran et al 1994). Furthermore, most preservatives induce occasional dermal irritation (Adams & Mailbach 1985; deGroot et al 1986, 1988; deGroot & Bruynzeel 1988).

The antimicrobial activity of small quantities of silver ions in aqueous media has been known for a long time (von Naegeli 1893), and many authors (Romans 1954; Woodward 1963; Holden 1970) have studied water disinfection using silver. More recently, electrically-generated silver ions have been widely employed to decontaminate drinking water. At the active concentration ($0.05\,mg\,L^{-1} \equiv 4{\cdot}6 \times 10^{-7}$ M), no adverse effects were apparent in laboratory animals (Schmuter et al 1987).

In a recent paper, the antimicrobial activities of $AgNO_3$, AgCl and Ag ions obtained through electrochemical methods were compared. We demonstrated that anodic silver ions showed highest activity also when challenged with large inocula (Simonetti et al 1992). This microbicidal activity appeared particularly enhanced at higher temperatures.

The wide antimicrobial spectrum, the high microbicidal potency, the good water solubility and the safety of anodic silver, therefore, provide an encouraging background to the investigation of the use of this ion as a preservative in pharmaceutical or cosmetic formulations.

We now report the antimicrobial activity of electrochemical Ag^+ ($\approx 10^{-6}$ M) in aqueous solutions of nonionic surfactants as well as in heterogeneous systems. Nonionic surfactants are well-known inhibitors of the antimicrobial activities of many preserving agents (Patel & Kostenbauder 1956; Barr & Tice 1957; Jacobs et al 1975), and they are also growth promoters of Gram-negative bacteria, particularly pseudomonads (Bean et al 1965; Bryce & Smart 1965). Several nonionic surfactants, showing substantial differences in chemical structure, were therefore tested in aqueous solution and in dispersions containing almond oil, as a model lipid phase.

Botanical extracts are present in many cosmetic products and in some pharmaceutical preparations. These often interfere with preserving agents or support microbial growth (Croshaw 1977; Steinberg 1991; Moral 1992). To evaluate possible interference with the antimicrobial activity of anodic silver, botanical extracts were added to the model dispersions in some experiments.

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Materials and Methods

Purified water was used for all solutions. Each aqueous solution was sterilized by filtering through a 0·22-μm paper filter (Kostar Corp.). Electrochemical silver solutions were prepared using a 4·5-V battery connected to two cylindrical silver electrodes (length, 8 cm; cross section, 4 mm^2; distance between electrodes, 3 cm) in a 100-mL glass cylinder. In all samples, the Ag^+ concentration was determined, as previously reported (Simonetti et al 1992), by a potentiometric method using an Ag_2S electrode. Silver ion concentration was adjusted to 10^{-6} M in each experiment. All silver solutions were protected from light. All experiments were performed at 25° C. The surfactant concentration in aqueous samples ranged between 0·5 and 7% w/w. The composition (w/w) of the model dispersion was: almond oil 53%, cetyl alcohol 4% (to increase dispersion stability), surfactants 1% (Tween 60/Span 60 3·16:1 or Renex 648/Renex 678 2·57:1), botanical extract (powder) 1·5%, aqueous solution of Ag^+ (10^{-6} M) to a total volume of 100. The pH was adjusted to 7·0 with citrate buffer 0·01 M. Tween and Span were purchased from Fluka Chemika AG Switzerland, Renex from ICI America Inc. Lipophilic materials and botanical extracts (Taraxaco, *Centella asiatica*, *Fucus vesciculosus*, *Aloe vera*) were from Agrar (Soc. Industr. Comm. Rome, Italy). Brain heart infusion (BHI), Sabouraud dextrose agar and TSA broth were from BBL (Beckton Dickinson Microbiology System, Cockeyville, USA).

Microbial strains

Escherichia coli 229, *Pseudomonas aeruginosa* 145, *Staphylococcus epidermidis* 5 and *Candida albicans* FE 53 were from the collection of the Microbiology Institute, Faculty of Pharmacy, University of Rome. All microbial strains were isolated from various clinical specimens and identified by standard methods. For the inoculum preparation and for viable cell counting, bacteria were grown in BHI agar for 24 h at 37° C, *C. albicans* was grown in Sabouraud dextrose agar for 48 h at 37° C. The microbial cells were washed and resuspended in water containing potassium dipotassium phosphate (0·025%, pH ≈ 7·4) before use. The cell suspension concentration was adjusted to ≈ 10^8 cells mL^{-1} using optical density at 540 nm, measured on a Perkin-Elmer 552 spectrophotometer.

To dilute test samples for viable counting, a solution (solution E) obtained from TSA broth (Trypticase peptone (1·7 g), phytone peptone (0·3 g), sodium chloride (0·5 g), dipotassium phosphate (0·25 g), dextrose (0·25 g), water (1000 g)) and Tween 20 (50 mL), according to Lucas (1978), was employed.

Survivor–time curves

The microbicidal activity was determined at 25° C. Survival curves were obtained by linear regression of the log of surviving microorganisms as a function of the time after inoculation into the test system. The D-value (time required to achieve 90% reduction of viable cells) was also calculated (Orth 1979) for each microorganism in each experiment. Experimental points, in all the experiments, covered at least 4 log cycles of viable counts.

Aqueous solutions. Each sample of 9-mL silver-surfactant solution received 1 mL microbial suspension. Immediately after inoculation and at each sampling time, 0·1 mL sample was diluted 1:100 with solution E to arrest the silver activity. To perform viable counts, each sample was serially diluted by factors of ten and colony-forming units were counted, on plates containing 30–300 colonies, after incubation on appropriate media.

Dispersions. Two hundred grams of model dispersion received 20 mL microbial suspension to give an initial viable count of 10^7-10^8 cells mL^{-1}. Immediately after inoculation and at each sampling time, a sample of ≈ 10 g dispersion was weighed, and mixed with 1 mL mineral oil, 1 mL Tween 80 and diluent E to obtain a 1 in 10 dilution. An aliquot of this sample (0·1 mL) was further diluted with 9·9 mL diluent E. To perform viable counts each sample was serially diluted and colony-forming units were counted on plates containing 30–300 colonies after incubation on appropriate media.

Statistical analysis

To test if the slopes (β) of regression curves of two different experiments were significantly different (parallel regression), a *t*-test was used (Armitage 1975). All statistical analyses were at 5% level of significance.

Results

The silver ion concentration of a 10^{-6} M solution of anodic silver remained unchanged after addition of Tween 20 (7% w/w), Renex 690 (7% w/w)) or botanical extracts (1·5% w/w), which were the highest concentrations of these reagents used in the experiments. Aqueous solutions of Tween 20 or Renex 690 were tested against *E. coli*, *P. aeruginosa*, *S. epidermidis* and *C. albicans* and showed no microbicidal activity or growth effect.

To evaluate the possible microbiological interferences of nonionic surfactants on silver activity, for each surfactant, seven aqueous samples of 10^{-6} M anodic silver, containing different concentrations of Tween 20 or Renex 690 were tested against *E. coli*, *P. aeruginosa*, *S. epidermidis* and *C. albicans* and the regression equations of survival curves were computed. In all experiments for both Renex 690 and Tween 20, the *t*-test carried out among regression coefficients (β) showed that the interfering effect, when present, was independent of surfactant concentration. In Table 1 we report the regressions obtained for each strain and, as reference, the regression equations related to the activity of Ag^+ 10^{-6} M. By comparing the slopes (β) obtained from surfactant and reference silver solutions, it appeared that Renex 690 did not alter silver antimicrobial potency apart from *P. aeruginosa*, for which the rate of kill was reduced to about 0·8 times with respect to the reference. Tween 20 caused a strong reduction of rate of kill to about 0·65, 0·55 and 0·51 times, respectively, for *E. coli*,

Table 1. Microbial activity against *E. coli*, *P. aeruginosa*, *S. epidermidis* and *C. albicans* of 10^{-6} M anodic silver aqueous solutions containing Tween 20 or Renex 690.

	Intercept of regression curve	Slope of regression curve (β)	Correlation coefficient from regression (R^2)	D-values (min)
Tween 20				
E. coli	7·590	−0·0587	0·975	17·0
P. aeruginosa	7·540	−0·0562	0·985	17·8
S. epidermidis	7·484	−0·0621	0·987	16·1
C. albicans	7·376	−0·0519	0·982	19·2
Renex 690				
E. coli	7·394	−0·0920	0·993	10·9
P. aeruginosa	7·379	−0·0859	0·991	11·6
S. epidermidis	7·404	−0·0677	0·990	14·8
C. albicans	7·185	−0·0940	0·983	10·6
Ag^- 10^{-6} M				
E. coli	7·328	−0·0920	0·995	10·8
P. aeruginosa	7·481	−0·1109	0·991	9·0
S. epidermidis	7·240	−0·0689	0·985	15·0
C. albicans	7·142	−0·0942	0·990	10·6

Table 2. Microbial activity (D-values, in min), against *E. coli*, *P. aeruginosa*, *S. epidermidis* and *C. albicans*, of Ag^+ 10^{-6} M in different experimental conditions.

	E. coli	*P. aeruginosa*	*S. epidermidis*	*C. albicans*
Dispersions prepared with Tween 60/ Span 60	19·8	18·5	19·1	22·2
Dispersions prepared with Renex 648/ Renex 678	12·1	12·0	16·8	12·1
Dispersions prepared with Tween 60/ Span 60 containing Taraxaco	67·6	71·4	69·9	76·3
Dispersions prepared with Tween 60/ Span 60 containing *Centella asiatica*	73·5	69·4	72·5	74·0
Dispersions prepared with Tween 60/ Span 60 containing *Fucus vesciculosus*	73·5	80·0	69·0	84·7
Dispersions prepared with Tween 60/ Span 60 containing *Aloe vera*	76·3	73·5	70·9	80·6
Aqueous solution of 10^{-6} M anodic silver	10·8	9·0	15·0	10·6

P. aeruginosa and *C. albicans*, while *S. epidermidis* was reduced to a lesser extent (0·76 times). In all cases, the rate of kill by anodic silver appeared very high because a reduction of more than 4 log units of the microbial concentration was observed in each test sample. In all samples, the concentration of viable cells 24 h after inoculation, resulted in less than 0·01% of the starting concentration.

Table 2 summarizes the D-values related to the antimicrobial activity of Ag^+, tested against *E. coli*, *P. aeruginosa*, *S. epidermidis* and *C. albicans*, assayed in two oil/water dispersions stabilized with Tween 60/Span 60 or Renex 648/Renex 678 and in some oil/water dispersions stabilized with Tween 60/ Span 60 containing botanical extracts commonly employed in cosmetic preparations.

By comparing the D-values reported in Tables 1 and 2, the rate of kill of silver ion can be seen to be unaffected by the lipophilic phase. However, as expected, the rate of kill was strongly diminished in the presence of botanical extracts.

To evaluate the persistence of the antimicrobial activity of Ag^+ in these experimental conditions, the dispersions containing botanical extracts were repeatedly inoculated (time 0, day 3, day 5, day 7). After each inocula or re-inocula, the cell concentration, in each sample, was $\approx 10^8$ cells mL^{-1}. The number of microorganisms surviving 24 h after seeding was compared with that present immediately after each inoculation. In all samples, the concentration of viable cells 24 h after inoculation resulted in less than 0·01% of the starting concentration.

The antimicrobial activity of anodic silver against each strain in the different experimental conditions is summarized in Figs 1–4.

To evaluate anodic silver as a cosmetic preservative, we carried out a preliminary test to verify any adverse skin reaction to Ag^+. Patch tests with a 10^{-6} M silver solution were performed on the forearms of ten volunteers: the left forearm was previously cleaned with a common detergent and the right one with acetone. No skin interaction was observed after 24 or 48 h on either forearm.

Discussion

According to the linear regression method proposed by Orth (1979) as a rapid procedure to evaluate preservatives

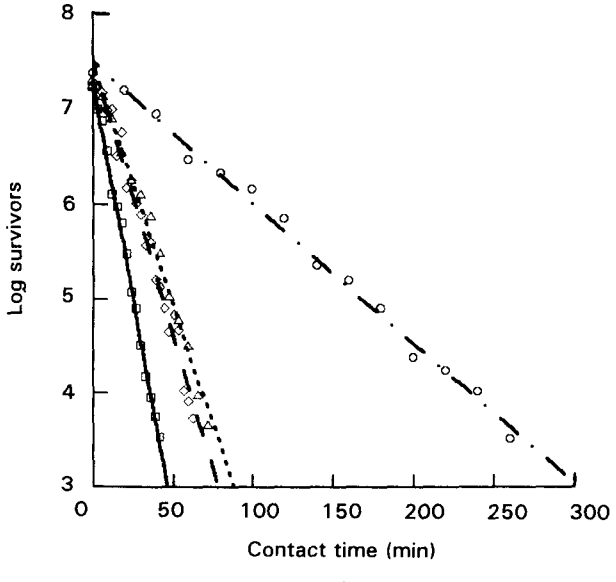

FIG. 1. Antimicrobial activity of 10^{-6} M Ag^+ (□) against *E. coli* in the presence of 3% Tween 20 (△), dispersion prepared with Tween 60/Span 60 (◇), and this dispersion with added Taraxaco (○).

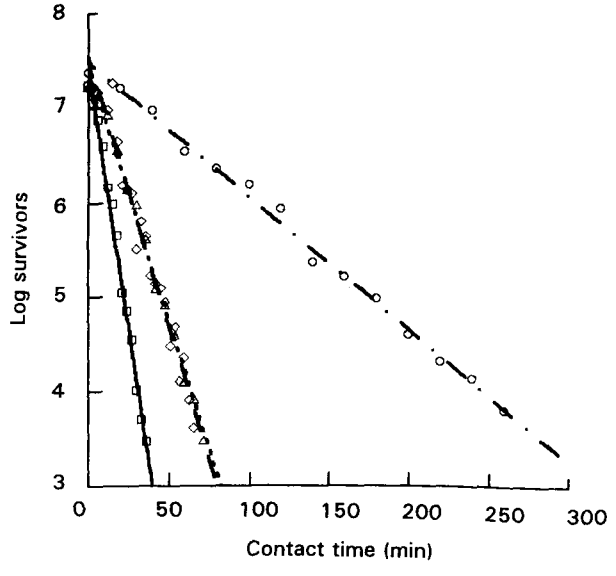

FIG. 2. Antimicrobial activity of 10^{-6} M Ag^+ (□) against *P. aeruginosa* in the presence of 3% Tween 20 (△), dispersion prepared with Tween 60/Span 60 (◇), and this dispersion with added Taraxaco (○).

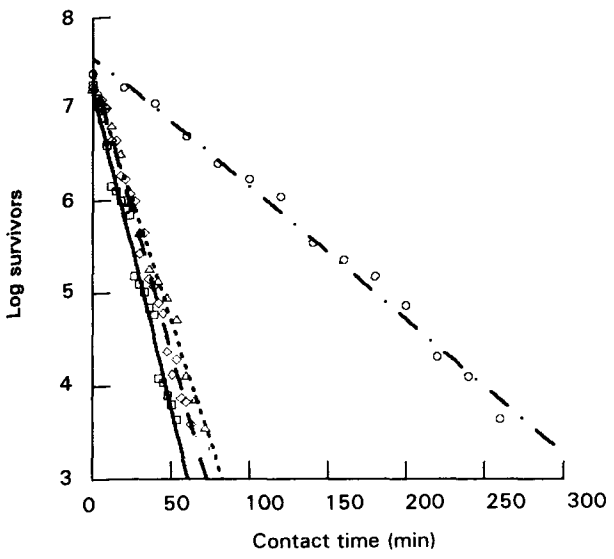

FIG. 3. Antimicrobial activity of 10^{-6} M Ag^+ (□) against *S. epidermidis* in the presence of 3% Tween 20 (△), dispersion prepared with Tween 60/Span 60 (◇), and this dispersion with added Taraxaco (○).

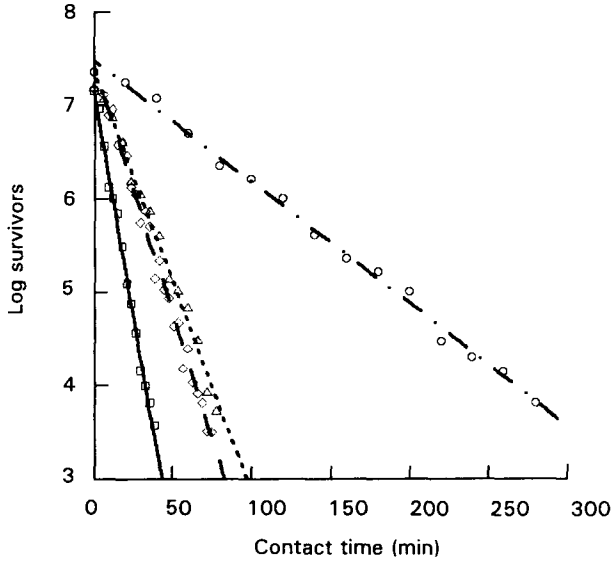

FIG. 4. Antimicrobial activity of 10^{-6} M Ag^+ (□) against *C. albicans* in the presence of 3% Tween 20 (△), dispersion prepared with Tween 60/Span 60 (◇), and this dispersion with added Taraxaco (○).

efficacy, a short D-value is desirable for a good preservative agent. In particular, Orth recommends a D-value < 4 h for pathogenic microorganisms and 28 h or less for non-pathogenic bacteria or fungi.

From the data reported, the D-values for all experimental conditions are remarkably lower than the suggested thresholds. An eloquent evaluation of silver microbicidal potency is easily accomplished by comparing the experimental D-values, respectively 294, 156, 131 and 128 min, related to the microbial activity of aqueous saturated solutions of methylparaben against *E. coli*, *P. aeruginosa*, *S. epidermidis* and *C. albicans*. The parabens were used as positive reference as they are the most preferred preserving agents, particularly in cosmetics (Steinberg 1992).

The high rate of kill of anodic silver is very useful to ensure a rapid reduction of microorganisms. However, the effectiveness of silver in keeping the number of surviving organisms at less than 0·01% of the starting inoculum after repeated inocula, even in the presence of strong interfering additives, appears the most interesting feature for its possible use as a preserving agent in multiple-dose products.

The usefulness of anodic silver as a preserving agent is also supported by the fact that the increase of microbicidal activity at raised temperatures (Simonetti et al 1992), could be very useful, for instance in the cosmetic or pharmaceutical industries, to ensure a more rapid drop of microbial concentration during product manufacture. From a toxicological point of view, electrochemical silver solutions have to be considered safe at least at the silver concentrations considered.

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